Nisin induced morphological changes and disruption of growth in *Bacillus subtilis*

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DECLARATION

To the best of my knowledge the research project report entitled "**Nisin induced morphological changes and disruption of growth in *Bacillus subtilis***" reported here in its original and has been submitted to National Institute of Technology, Rourkela for partial fulfilment of the degree of Master of Science in Life Science is a bonafide record of the project work carried out by me under the supervision of Dr. Mohammed Saleem, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. This thesis is my own work and that, to the best of my knowledge and belief, the matter and results of this thesis has not been submitted by any other research persons or any students.

I do hope, this project work will satisfy our beloved teachers. I solicit kind and favourable appreciation.

Eva Dash

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CERTIFICATE

This is to certify that the thesis entitled "**Investigation of antimicrobial potential of Nisin against *Bacillus subtilis***" which is being submitted by **Ms Eva Dash**, Roll No. **413LS2029**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Saleem 11/5/15

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Contents:

ABSTRACT

Nisin is a 34 residue long cationic lanthionine antibiotic produced by *Lactococcus lactis* and known to show antimicrobial activity against a broad spectrum of gram-positive bacteria. During its antimicrobial action it is known to target intermediates in the bacterial cell wall biosynthesis, lipid II, and undecaprenyl pyrophosphate. Recent discoveries of lipid II as a target for nisin has brought nisin to the forefront, as a model case, in the battle against antibiotic resistance and assessing the combination of using conventional antibiotics with nisin remains to be explored. Here we evaluated the effect of antimicrobial activity of Nisin on *Bacillus subtilis* by determining the MIC (Minimum inhibitory concentration), MBC (Minimum bactericidal concentration), Zeta potential (electrokinetic potential), SEM (Scanning electron microscopy), FESEM (Field emission scanning electron microscopy) and bacterial cell viability by Baclight Fluorescencein the presence and absence of Ampicillin. We observe that increasing concentrations of Nisin drastically prolong the lag phase of *Bacillus subtilis* and cause excessive delay in reaching the stationary phase. The electron micrographs depict rupturing of bacterial cell membrane, significant loss of surface area and volume. Further, an interesting transformation of cylindrical *B subtilis* into ellipsoidal shape is observed.

Chapter 1
INTRODUCTION

1. INTRODUCTION:

All organisms protect themselves from microorganisms and the ability of preventing the onset of infection is dependent on the innate immune response. Antimicrobial peptides (also known as host defense peptides) are part of innate immune system and are present in all living classes. These cytosolic peptides serve organisms for both offensive and defensive purposes. They can be instantly harmful to the broad range of microbes and have additional activities that impart a marked change on quality and effectiveness of innate responses and inflammation. Furthermore, the unique mode of action of antimicrobial peptides is helpful to produce new anti microbial drugs as the bacteria become resistant to the conventional antibiotics[1].

Hundreds of antimicrobial peptides have been isolated so far and irrespective of their origin, structure and function, most of these peptides have similar properties. They are generally composed of <60 amino acid residues (mostly common L-amino acids), having net positive charge. They are amphipathic and mostly they are membrane active. When the antimicrobial peptides interact with the membranes, they alter the organization of bilayer and make it permeable, which is caused by membrane depolarization. Hence the interaction of antimicrobial peptides with membrane involving electrostatic and hydrophobic interaction is necessary precursor to cell death. And of course, some antimicrobial peptides are found to interact with the host cell directly to stimulate host gene products such as chemokines, chemokinereceptors, integrins, transcriptional factors [2].

1.1 Antimicrobial resistance:

Antimicrobial resistance (AMR) happens when an organism obtains a hereditary transformation making it impervious to the impact of one or more antimicrobial operators that were once viable. This hereditary change may be procured suddenly or by quality exchange. Microorganisms can get to be impervious to anti-toxins, for which the term anti-infection resistance is utilized. Organisms that are impervious to different antimicrobials are termed multidrug safe [3].

Antibiotic are vigorously used both in human and veterinary medicine and this causes the antibiotic resistance among this population. Use of antibiotics promotes the resistance in bacteria due to selection pressure to flourish and the vulnerable bacteria to die off. As resistance to antibiotics becomes more common, a greater need for alternative treatments arises. Although there is a need for new antibiotic therapies, newly approved drugs become very less in number.[4,5,6]. Common types of drug-resistant bacteria include methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant S. aureus (VRSA), extended spectrum beta-lactamase (ESBL), vancomycin-resistant Enterococcus (VRE) and multidrug-resistant. There may be various cause for antibiotic resistance in bacteria like transfer of genes from other bacteria by horizontal gene transfer, genetic mutations, conjugation, uptake of foreign DNA by

transformation, or transduction. Contamination by safe microorganisms may be group obtained or health awareness related [7,8,9].

Antibiotic resistance poses a grave and growing global problem: a World Health Organization report released April 2014 stated, "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. Antibiotic resistance—when bacteria change so antibiotics no longer work in people who need them to treat infections—is now a major threat to public health."

1.2 Activities of antimicrobial peptides:

There are varied modes of action of antimicrobial peptides and is different for different bacterial species. They frequently target the cytoplasmic membrane but they also interfere with DNA and protein synthesis, protein folding and cell wall synthesis. Bacterial surfaces are mostly charged may be anion or hydrophobic so this facilitates the interaction between peptide and the bacterial surface. Their amino acid composition, amphipathicity, cationic charge and size permit them to connect to and embed into layer bilayers to shape pores by 'barrel-stave', or 'carpet' mechanisms. There may be mechanism in which they bind to intracellular molecules which are essential for cell growth. This mechanism can involve inhibition of cell wall synthesis, deformation in membranes, autolysin activation, inhibition of synthesis of proteins, DNA and RNA restricting activity of certain enzymes. Exact mechanism of killing bacteria is remained to be explored. This can be achieved by determining antimicrobial activity of these by calculating the minimal inhibitory concentration (MIC), which is the lowest concentration of drug which inhibits bacterial growth [10].

1.3 Factors determining antimicrobial efficiency:

There are various factors are associated with antimicrobial peptides among which the net charge on peptide contributes most, which is mostly cationic. Bacterial membrane surface compared to mammalian cell surface charge so that Antimicrobial peptides have different affinities for both type of membranes.

In bacterial cell membrane there is absence of cholesterol so it helps antimicrobial peptides to interact with cell membrane as compared to mammalian cell in which cholesterol obstructs the interaction. Cholesterol stabilizes the lipid bilayer of mammalian membrane which may be the cause of reduced interaction between peptide and membrane.

Transmembrane potential have also a key role in peptide lipid interactions. There is an inside-negative transmembrane potential existing from the outer leaflet to the inner leaflet of the cell. This charge difference causes the transmembrane potential to negative. This negative charge

facilitates the permeabilization of membrane by positively charged peptides. Therefore bacterial membranes are more prone to be attacked by cationic antimicrobial peptides.

Electrostatic interactions may also be decreased by increasing the ionic strength of bacterial membrane Similarly, it is also believed that increasing ionic strength, which in general reduces the activity of most antimicrobial peptides, contributes partially to the selectivity of the antimicrobial peptides by weakening the electrostatic interactions required for the initial interaction.

1.4 Mechanism of antimicrobial activity:

The bacterial cell membranes are rich in acidic phospholipids, such as phosphatidylglycerol and cardiolipin. These phospholipid headgroups are highly negatively charged. Therefore, the outer most leaflets of the bilayer that is exposed to the outside of the bacterial membranes are more attractive to the attack of the positively charged antimicrobial peptides. So the interaction between the positive charges of antimicrobial peptides and the negatively charged bacterial membranes is mainly electrostatic in nature. Moreover, there are also some hydrophobic interactions between the hydrophobic regions of the antimicrobial peptides and the zwitterionic phospholipids (electrically neutral) surface of the bacterial membranes as antimicrobial peptides form structures with a positively charged face as well as a hydrophobic face.

1.5 Diversity in antimicrobial peptide:

More than 500 antimicrobial peptides have been discovered from animals and plants so far. However, the distribution is so vast that no similar peptide sequence is recovered from two different even if closely related species.

The whole set of antimicrobial peptides are divided into four different groups[1] as described below.

GROUP I:

(Linear peptides with an α-helical structure):

This group is one of the larger and most studied groups of antimicrobial peptides. It forms cationic amphipathic helices, e.g. magainin, cecropin-A, temporinsetc. These peptides adopt disordered structures in aqueous solution while fold into a α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-Helical peptides are often found to be

amphipathic and can either absorb onto the membrane surface or insert into the membrane as a cluster of helical bundles.

GROUP II:

(Conformationally more restrained peptides, predominantly consisting of *β*-strands connected by intramolecular disulfide bridges):

β-sheet peptides are cyclic peptides constrained either by disulfide bonds, as in the case of human β-defensin-2, or by twisting of the peptide backbone, as in the case of gramicidin S, tyrocidines. They largely exist in the β-sheet conformation in aqueous solution that may be further stabilized upon interactions with lipid surfaces.

GROUP III:

(Linear peptides with an extended structure, characterized byoverrepresentation of one or more amino acids):

Certain antimicrobial peptides have an unusual amino acid composition, having asequence that is rich in one or more specific amino acids. For example, the peptidehistatin, which is produced in saliva, is highly rich in His residues.This peptide translocates across the yeast cell membrane and targets mitochondria by an unusual antifungal mechanism.

GROUP IV:

(Peptides containing a looped structure):

Lantibiotics are a class of peptide antibiotics that contain the characteristic polycyclic thioether amino acids, lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid.One of the lantibiotics, nisin, iscurrently used as an antimicrobial agent for food preservation and this peptide hasrelatively high activity against Gram-positive bacteria due to its specific high affinitywith Lipid II, a precursor in the bacterial cell wall synthesis.Lantibiotics show substantial specificity for some components (e.g., lipid II) of bacterial cell membranes especially of Gram-positive bacteria. Type A lantibioticskill rapidly by pore formation; type B lantibiotics inhibit peptidoglycan biosynthesis. They are active in very low concentrations.

1.6 Why we choose Nisin?

Nisin:

Nisin is a 34-residue-long peptide having antimicrobial activity against Gram-positive bacteria. It contains the uncommon amino acids lanthionine, methyllanthionine, didehydroalanine, and didehydroaminobutyric acid. It is positively charged and is amphipathic. Nisin is post-translationally modified such that the serine and threonine residues are dehydrated to become dehydroalanine and dehydrobutyrine.

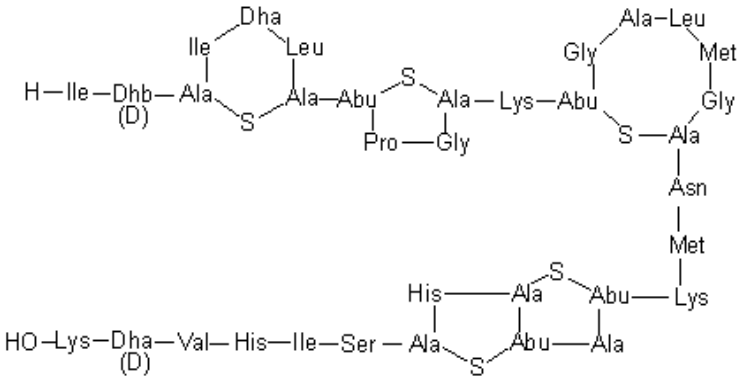


Fig 1: Structure of Nisin [fa.kfda.go.kr]

It is not pathogenic to humans, as it has been used as food preservative.

The treatment of nisin results in rapid efflux of small cytoplasmic compounds. It is accepted that nisin targets bacterial plasma membrane and it kills the cell by pore formation that leads to the collapse of vital ion gradients.

The precise mechanism of mode of action of nisin is not known yet. It may lead to pore formation or insertion or disruption of cell wall synthesis or depolarization of cell membrane. However, the mechanism leading to pore formation by nisin is hypothesized to occur into different steps. The first step is the binding to target membrane, followed by insertion into lipid phase of membrane that finally leads to pore formation[11] .

Nisin binding:

Nisin binds preferably to membrane containing anionic lipids that should be present in relatively large amount that is very common in Gram-positive bacteria[3].

Nisin insertion and orientation:

The amphipathic properties allow nisin to insert into the lipid phase of cell membrane. The anionic phospholipids are essential for efficient insertion of nisin. Nisin variants with either extensions oat N-terminus, or with minor changes in the first ring severly reduce the ability to

insert in the lipid monolayer where C-terminus does take part in the insertion. Hence, it is clear that the N-terminus of the nisin insert first. It is also proved that an increase in the amount of antibiotic lipids results in the increased depth of insertion of nisin molecule. The stable orientation of nisin in the membrane is parallel with respect to the membrane surface.

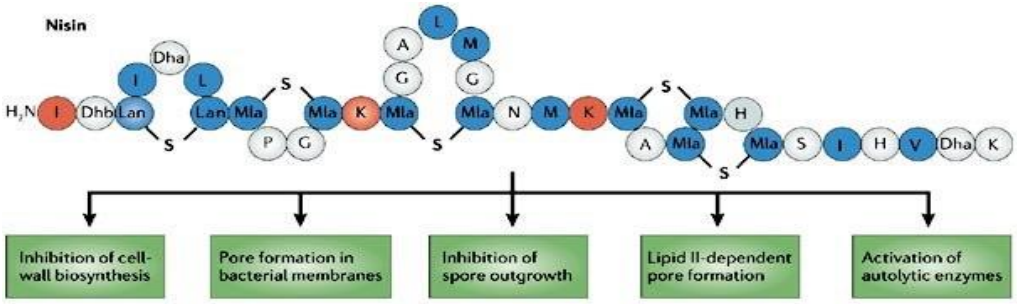


Fig 2: Structure of Nisin: The residues in red have a positive net charge, those in blue are hydrophobic. The amino terminus is indicated with NH2. Dha, dehydroalanine; Dhb, dehydrobutyrine; Lan, lanthionine; Mla, methyllanthionine; S, thioether bridge [www.natures.com].

Pore formation:

The amount of bound nisin greatly influences the amount of leakage that occurs. The size of the nisin pore is supposed to be 1 nm.

1.7 Bacteria:

Bacteria are of a large domain of prokaryotic microorganisms. Typically a few micrometres in length, bacteria have a number of shapes, ranging from spheres to rods and spirals. Bacteria were among the first life forms to appear on Earth, and are present in most of its habitats.

On the basis of the cell wall composition and its stain ability with Gram stain, bacteria are classified into two groups such as, Gram-positive and Gram-negative bacteria.

Gram-positive bacteria:

This type of bacteria remains stained by with Gram staining even after washing alcohol or acetone. Here outer cell membrane is absent and cell wall is 20-30 nm thick. The cell wall contains teichoic acid and 70-80% murein where lipid content is very low.

Eg., *Staphylococococcus spp*., *Enterococcus spp*., *Bacillus spp*.

Gram-negative bacteria:

These bacteria do not retain the colour of the stain after washing with alcohol. Here outer membrane is present. Cell wall contains less murein where lipid content is high of about 20-30%. Most of the pathogenic bacteria belong to this group.

Eg. *Escherichia spp, Pseudomonas spp., Salmonella spp*.

1.7.1 Why we chose *Bacillus subtilis?*

Bacillus subtillis:

Bacillus subtilis, which is overall known as the **Hay bacillus** or **Grass bacillus**, is a Gram-positive, catalase-positive bacterium. It is found in soil, the gastrointestinal tract of ruminants and people. It is a bar molded bacterium, and can structure an intense, defensive endospore, permitting the life form to endure amazing ecological conditions. *B. subtilis* has generally been delegated a commit aerobe, however proof exists that it is a facultative aerobe [4].

Pathogenesis:

The distinctive types of *Bacillus* deliver a mixed bag of extracellular items including antimicrobial substances, proteins, colors, and poisons in couple of species [11]. Catalysts that can be found on society incorporate amylase, collagenase, hemolysin, lecithinase, phospholipase, protease, and urease. Two distinct sorts of enterotoxins are delivered by *B. cereus* amid exponential development: the enterotoxin bringing on the runs and the emetic poison. Late studies on the visual harmfulness elements of *B. cereus* showed that hemolysin BL, a tripartite dermonecrotic penetrability element and rough exotoxin arrangements in vivo, brought about endophthalmitis clinically normal for the organic entity inside 4 hours of immunization [12]. *Bacillus cereus* may have three different forms of enzyme activity related to lecithinase like activity; phosphatidylcholine hydrolase is the most studied form and frequently referred to as phospholipase C. This enzyme may have a secondary role in ocular infections by disrupting host cell membrane phospholipids exposed by the action of other toxins.

1.8Antibiotics:

Antibiotics are a type of antimicrobial used to treat various infections and used separately against bacteria. They may kill or reduce the growth of bacteria. For the rest of this dissertation we would only be interested in Ampicillin as *B. subtilis* is known to show Ampicillin resistant.

Ampicillin:

The antibiotic, Ampicillin, treats a number of bacterial infections. It comes under aminopenicillin family. Its activity is quite equivalent to that of amoxicillin. It is a beta-lactumantibiotic. It is an active against Gram-positive bacteria.

It is effective to respiratory and olfactory infections.

Mechanism:

Belonging to the penicillin group of beta-lactum antibiotics, ampicillin is able to penetrate Gram-positive and some Gram-negative bacteria. It differs from penicillin G, or benzylpenicillin, only by the presence of an amino group. That amino group helps the drug penetrate the outer membrane of Gram-negative bacteria.

Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell walls. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis; therefore ampicillin is bacteriocidal.

Chapter 2

METHODOLOGY

2. METHODOLOGY:

2.1 MIC (Minimum Inhibitory Concentration):

It refers to minimum inhibitory concentration. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum inhibitory concentration is helpful to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. It also helps determine the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents.

2.2 MBC (Minimal Bactericidal Concentration):

It stands for minimum bactericidal concentration.MBC refers to the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC.

2.3 CFU (Colony Forming Unit):

In microbiology, a colony-forming unit (CFU) is a unit used to estimate of the number of viable bacteria or fungal cells in a sample that has the ability to multiply by binary fission under the controlled conditions. Checking with CFU obliges refined the microorganisms and checks just feasible cells, interestingly with infinitesimal examination which numbers all cells, living or dead.

CFU can be calculated as,

(No. of colonies x dilution factor)/volume plated in mL.

2.4 ZETA potential (surface charge potential):

Colloidal particles dispersed in a solution are electrically charged due to their ionic characteristics and dipolar attributes.

Each particle dispersed in a solution is surrounded by oppositely charged ions called the fixed layer. Outside the fixed layer, there are varying compositions of ions of opposite polarities, forming a cloud-like area. This area is called the diffuse double layer, and the whole area is electrically neutral [13].

When a voltage is applied to the solution in which particles are dispersed, particles are attracted to the electrode of the opposite polarity, accompanied by the fixed layer and part of the diffuse double layer, or internal side of the "sliding surface". Zeta potential is considered to be the electric potential of this inner area including this "sliding surface". As this electric potential approaches zero, particles tend to aggregate [14].

If the particles acquire a large positive or negative zeta potential, they repel each other and form a stable suspension. Solutions are generally considered stable if they have a zeta potential of more than +30mV or less then -30mV. It should also be noted that zeta potential is highly dependent on pH. The point at which zeta potential reads 0mV is called the isoelectric point.

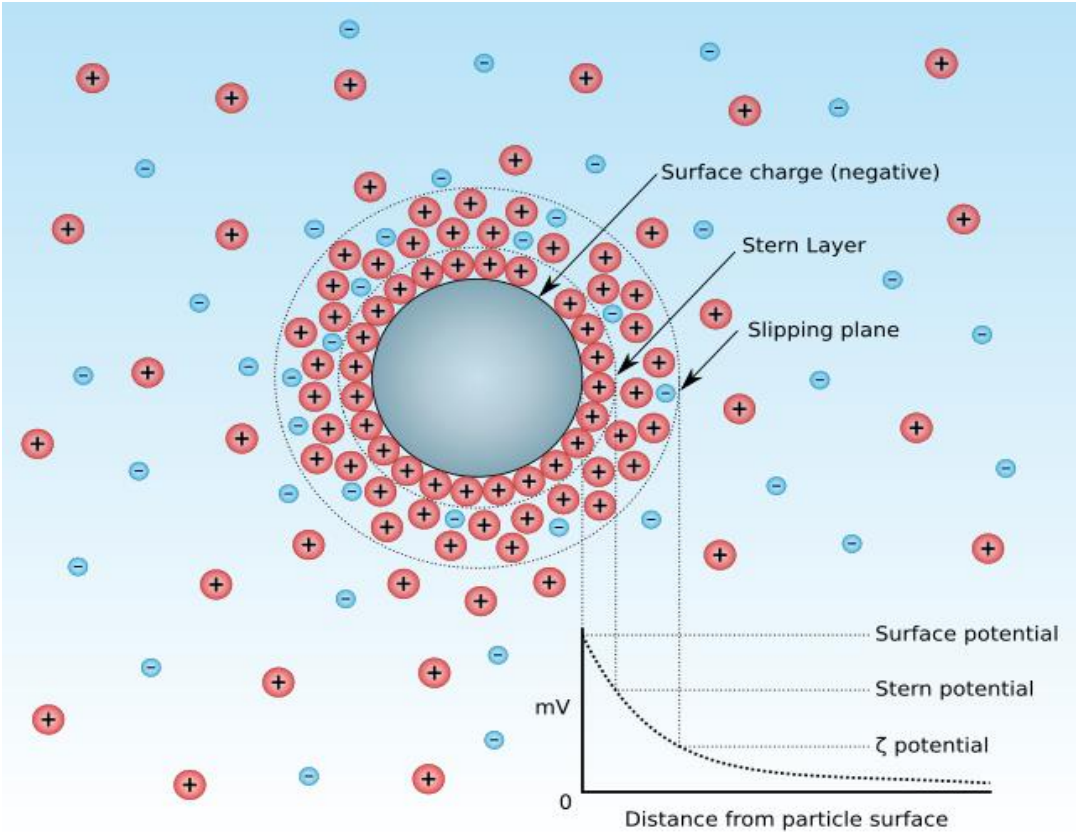


Fig 3: The ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium[en.wikipedia.org]

2.5 Electron microscopy:

Electron microscopy is utilized when the best determination is needed. Pictures delivered in an electron magnifying instrument uncover the ultrastructure of cells. There are two separate sorts of electron magnifying lens the transmission electron microscope(TEM) and the examining electron microscope(SEM). In TEM, electrons that go through the example are imaged. In SEM electrons that are reflected back from the example are gathered and surfaces of example are imaged.

What might as well be called the light source in an electron magnifying instrument is an'electron weapon'. At the point when a high voltage of 40,000 and 1,00,000 volts is gone between the cathode and anode, a tungsten fiber radiates electrons. The contrarily charged electrons go through a gap in the anode framing an electron pillar. The light emission goes through a stalk of electromagnetic lenses (the segment). Centering of the electron pillar is attained to by changing the voltage over the electromagnetic lenses. At the point when the electron pillar goes through the example a portion of the electrons are scattered while others are concentrated by the projector lens onto a bright screen or recorded utilizing photographic film. The electrons have constrained infiltration power which implies that the example must be thin (50-100nm) to permit them to go through.

Preparation of specimen:

For SEM& FESEM, samples are fixed in glutaraldehyde, dehydrated through a series of solvents and dried completely either by air or by critical point drying. The specimens are then mounted on a special metal holder or stub and coated with a thin layer of gold or platinum before viewing in the EM.

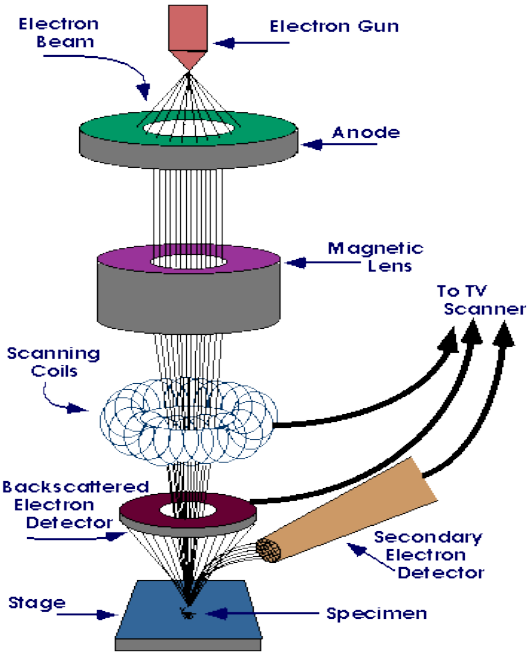


Fig 4: Workflow of Scanning Electron Microscopy [www.purdue.edu]

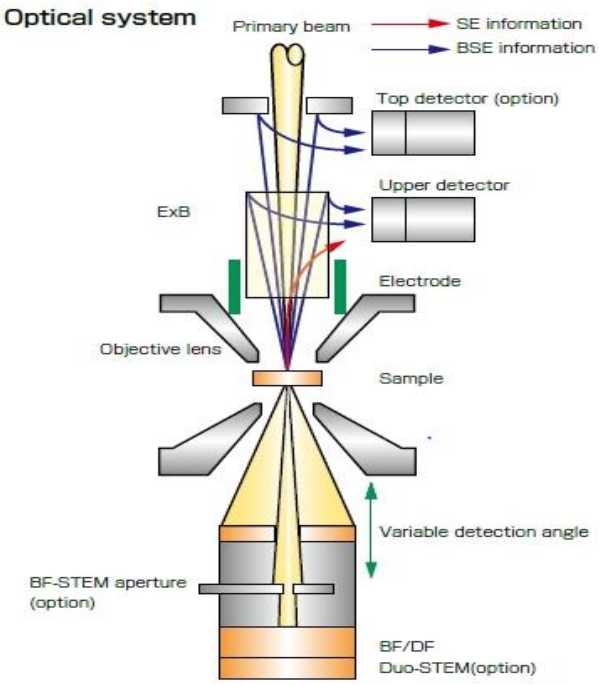


Fig 5: Workflow of Field Emission Scanning Electron Mocroscopy [www.goffinmeyvis.be]

2.6 BacLight cell viability assay:

LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen)was used to determine the bacterial cell viability. Conventional direct-count assays of bacteria viability are based on metabolic characteristics or membrane integrity.Cells with a compromised membrane that are considered to be dead or dying will stain red, whereas cells with an intact membrane will stain green[5].

It is compatible for bacterial cells and it detects images by fluorescence detection method. The commercially available LIVE/DEAD BacLight kit (Invitrogen) consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes. The emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer. Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between active and dead cells. While it seems accurate to assume that membrane-compromised bacterial cells can be considered dead, the reverse (that intact cells are active cells) is not necessarily true. Microscopic assessment of LIVE/DEAD-stained bacterial cells is usually simplified to either “green”-labeled (live) or “red”-labeled (dead) cells.

Chapter 3

EXPERIMENTAL PROCEDURE

3. EXPERIMENTAL PROCEDURE:

3.1 Preparation of HEPES buffer:

HEPES- 10mM (molecular weight- 238.30g/mol)

NaCl- 150mM (molecular weight- 58g/mol)

pH- 7.4

Volume- 500ml

HEPES:

$W = MW \times M \times V \div 1000$

$= 238.30 \times 10 \times 10^{-3} \times 500 \div 1000$

$= 1.1915g$

NaCl:

$W = 58.5 \times 150 \times 10^{-3} \times 500 \div 1000$

$= 4.38g$

Hence 1.1915g of HEPES buffer and 4.38g of NaCl were dissolved in milli Q (deionised) water. The volume was made upto 500ml. The pH is maintained at 7.4.

3.2 Media Preparation:

13g of nutrient broth was dissolved in 1000ml of water. To make the desired 75ml of the solution, the nutrient broth required was 13÷1000×75=0.975g.

0.975g of nutrient broth was weighed

It was dissolved in 75ml of distilled water in a measuring beaker

Three measuring conical flasks were taken

25ml of media was poured in each flask

The flasks were tightly plugged with cotton plugs

The flasks were wrapped with paper

They were autoclaved for 15 minutes

The media was ready to use

*1mg/ml antibiotic stock solution was prepared.

*To calculate the amount of Nisin required,

$C_1V_1 = C_2V_2$

Where,

$C_1 = 1000U/ml$

$V_1 = ?$

$C_2 = 50U/ml$

$V_2 = 300\mu l$

Hence, $V_1 = 15\mu l$

3.3 MIC (Minimum inhibitory concentration):

Stock solution of antibiotic and nisin (1mg/ml) were made and kept at 4*C.

Bacterial strain was inoculated in the media when it is cooled

It was incubated overnight (37*C,150 rpm) till O.D. reached at 1.0 at 600nm

Nisin was used in gradient concentration alone and with antibiotics in units of U/ml as 50 U/ml, 200 U/ml, 500 U/ml, 1000 U/ml and 1500 U/ml.

Ampicillin was injected 100 U/ml with increasing concentration of Nisin as above.

Incubate at 37º C for 8 hrs.

Finally, MIC was calculated for both Nisin and ampicillin in combination of Nisin.

3.4 Tabulation for MIC (Well-Plate preparation):

Sl no.	Solution (U/ml)	Nisin(*µ*l)	Culture(*µ*l)	Ampicilin(*µ*l)
1	Control	0	30	0
2	50	15	30	0
3	200	60	30	0
4	500	150	30	0
5	1000	60	30	0
6	1500	90	30	0
7	Amp-Control	0	30	30
8	50	15	30	30
9	200	60	30	30
10	500	150	30	30
11	1000	60	30	30
12	1500	90	30	30

3.5 MBC (Minimal Bactericidal Concentration):

96 well plate (prepared during MIC) was taken

⇩

10,000 times dillution

⇩

10ml solution was spread on agar plate

⇩

Incubation at 37° C for 8 hours

⇩

Colony counting

3.6 Zeta Potential Study:

Bacillus subtilis were allowed to grow overnight at 37*C and 150 rpm in 5ml MH broth

⇩

100µl of culture was inoculated in 5ml of MH broth

⇩

The suspension was then allowed to grow at 37° C for OD to be 1.0 (3×10^8cfu/ml)

⇩

1.5ml of the above culture was then taken and centrifuged at 13000 rpm for 8 minutes

⇩

Pellet was washed two times using HEPES buffer

⇩

100µl of nisin treated suspension was added to 900µl of *B. subtilis* cells

⇩

Zeta potential was measured after 1 hour

3.7 Scanning Electron Microscopy: (Slide Preparation)

1.5ml of overnight culture was taken

⇩

Centrifuged at 5000 rpm for 5 minutes

⇩

Pellets were collected and washed twice with PBS by centrifugation at 5000 rpm for 5 minutes

⇩

Pellets were suspended in PBS

⇩

One drop of suspended pellet was taken on glass slide

⇩

The glass slides were flooded by glutaraldehyde (2.5%) prepared in PBS

⇩

The slides were kept for overnight (15 hours approx.) incubation at 4° C

⇩

Washed with water

⇩

1% of tannic acid was flooded over slides

⇩

Kept for 5 minutes

⇩

Washed with distilled water

⇩

Slides were then subjected to a gradient ethanol wash sequentially as 30%, 50%, 70%, 90%, 100% Ehtanol

⇩

Slides were kept for drying

BacLight:

50mg/ml of nisin stock solution was prepared

⇩

For control 4.9ml broth and 100µl culture were taken in a falcon tube

⇩

For preparation of treated solution, 4.75ml of broth, 100µl culture and 150µl of nisin from the stock solution were taken in another falcon tube

⇩

Tubes were marked according to the concentration of nisin

⇩

Both the tubes were incubated overnight

⇩

Centrifugation at 7000rpm at 25*C for 15 minutes

⇩

Pellets were collected

⇩

500µl of HEPES buffer was added and mixed

⇩

5ml HEPES buffer were taken in 2 separate falcon tubes each

⇩

The pellets with 500µl buffer in the previous tubes were poured in the new falcon tubes

⇩

Centrifugation at 7000rpm at 25*C for 15 minutes

⇩

Pellets were collected

⇩

They are suspended in 5ml of HEPES buffer

⇩

Centrifugation at 7000rpm at 25*C for 15 minutes

⇩

Pellets were collected

⇩

2.5ml of HEPES buffer was added

⇩

333 µl culture from each falcon tubes were taken in separate eppendrof tubes

⇩

1µl BacLight dye was added in each tube

⇩

Incubation in dark for 15 minutes

⇩

5µl was taken on glass slide and covered with a cover slip

⇩

Image was seen in the Fluoroscent microscope

Chapter 4

RESULTS

4. RESULTS &OBSERAVATION:

4.1 MIC: (for 6 hours)

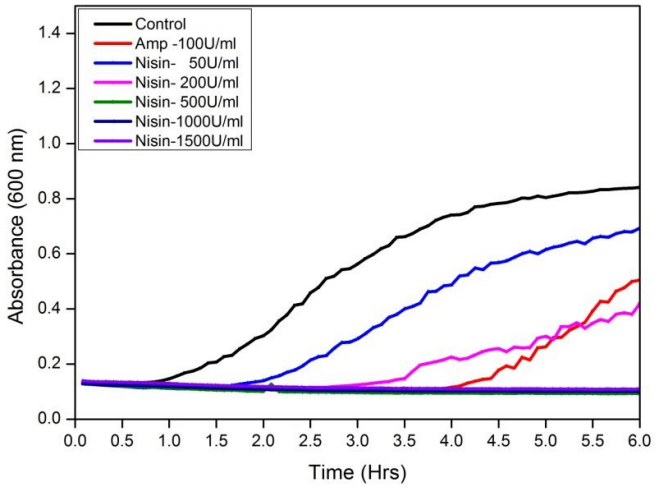


Figure 6: Showing effect of only nisin on bacterial growth in time v/s absorbance (OD) in 6 hours.

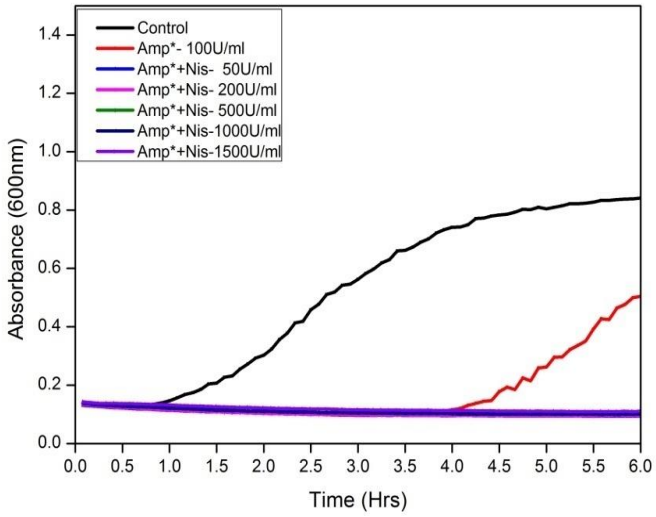


Figure 7: Showing effect of nisin with ampicillin on bacterial growth in time v/s absorbance (OD) in 6 hours.

4.2 MIC (for 20hours):

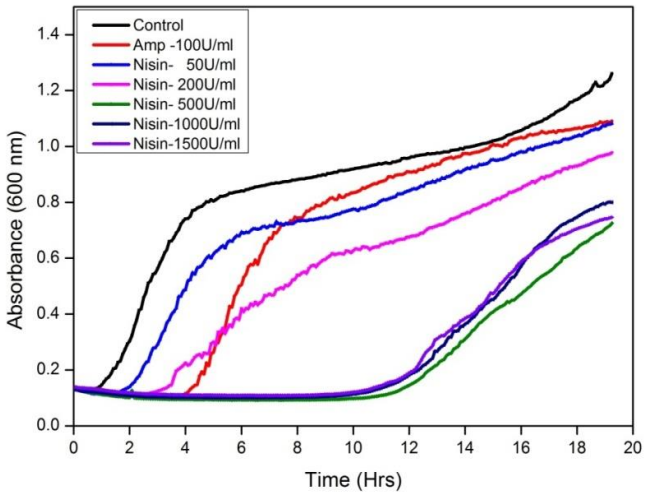


Figure8: Showing effect of only nisin on bacterial growth in time v/s absorbance (OD) in 20 hours.

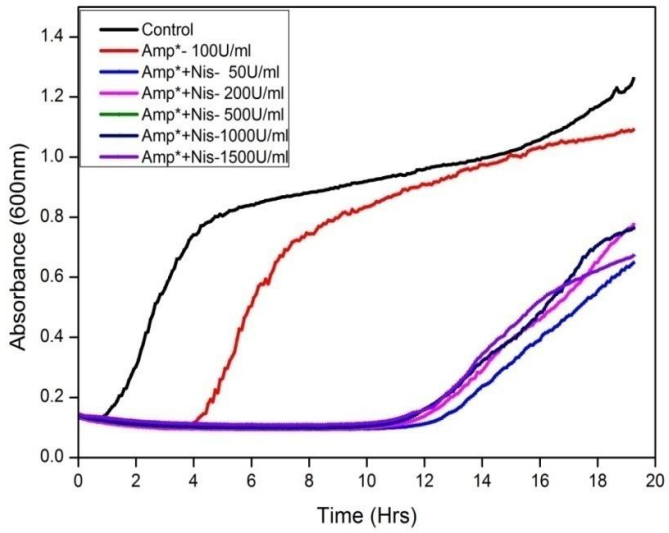


Figure 9: Showing effect of nisin with ampicillin on bacterial growth in time v/s absorbance (OD) in 20 hours.

Above MIC growth kinetics show prolongation of lag phase of bacterial growth in the presence of nisin and the same condition is observed when nisin is applied with ampicillin.

During lag phase, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. They are metabolically active and increase only in cell size.During this phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

In nisin (control) condition (Fig 6 and Fig 8), the bacteria grow freely as usual but in nisin (1500U/ml) condition (Fig 7 and Fig 9), the bacteria remain in stressed condition which reduces the efficiency of bacteria to synthesize enzymes, RNA, DNA etc required for their growth and metabolism.

4.3 MBC:

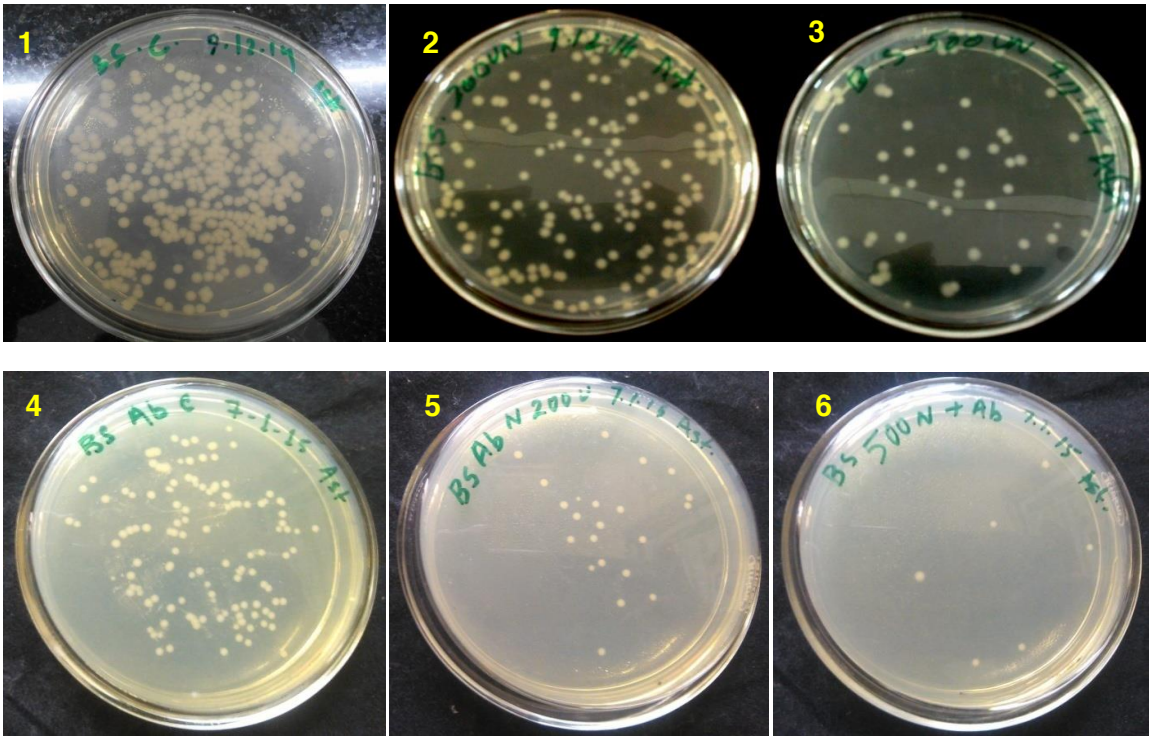


Figure 10: Images showing number of bacterial colonies formed in various concentration of nisn and ampicillin 1-Nisin (Control), 2-Nisin (200U/ml), 3-Nisin (500U/ml), 4-Ampicillin (Control), 5-Nisin (200U/ml)+Amp, 6- Nisin (500U/ml)+Amp

Tabulation (Depicting the colony count from the above images):

Sl. no	Plate	No. of bacterial colonies
1	Nisin (Control)	231
2	Nisin (200U/ml)	174
3	Nisin (500U/ml)	49
4	Ampicillin (Control)	91
5	Nisin (200U/ml)+Amp	22
6	Nisin (500U/ml)+Amp	07

As the concentration of nisin increases, number of bacterial colonies decreases and the same result is observed in case of nisin with ampicillin. The number of colonies reduces to about 5 folds when the nisin concentration increases control to 500 U/ml. That shows that nisin has antimicrobial effect that help reduce the number of colonies which is the result of inhibition of cell division.

4.4 : CFU:

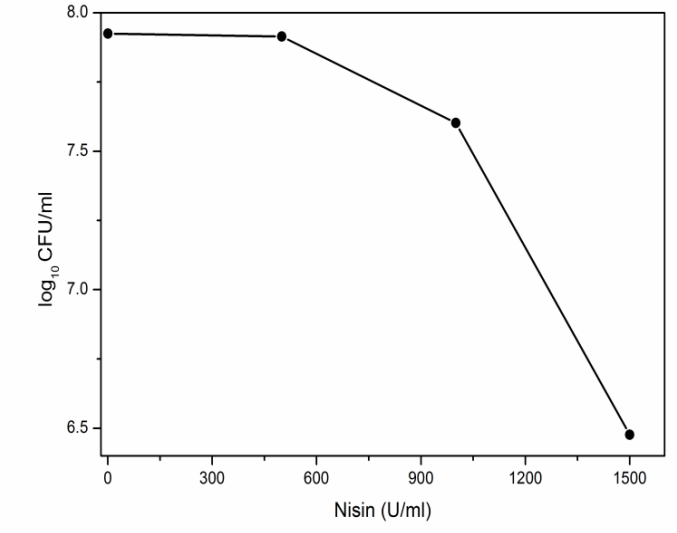


Figure 11: CFU/ml in log scale v/s Nisin concentration to determine the fold change in growth

Tabulation:

Sl. No	Plate	CFU/ml
1	Nisin (Control)	2.31×10^{7}
2	Nisin (200U/ml)	1.74×10^{7}
3	Nisin (500U/ml)	4.9×10^{6}
4	Ampicillin (Control)	9.1×10^{6}
5	Nisin (200U/ml)+Amp	2.2×10^{6}
6	Nisin (500U/ml)+Amp	7.0×10^{5}

As the concentration of nisin increases, cfu/ml decreases and the same result is observed in case of nisin with ampicillin.

CFU (colony forming unit) represents the number of viable bacteria. It shows the number of bacteria capable of living and reproducing to form a group of same bacteria. The graph above

shows reduction in bacterial colony with the increase in the nisin concentration tha t shows nisin is responsible for the reduction of the number of colonies.

4.5 Zeta Potential (Surface charge potential):

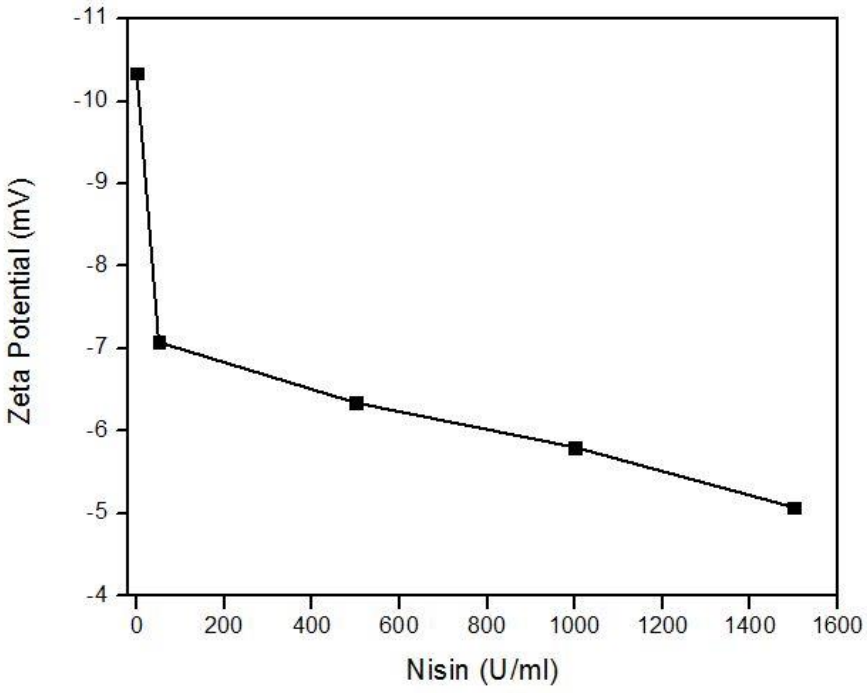


Figure 12: Zeta potential against concentatration of nisin

From the above graph we observed that, increasing concentrations of nisin shows a decrease in surface charge of *B. subtilis* which suggests neutralization of the surface potential by the interacting nisin. The change in membrane surface potential could lead to deformation of the bacterial membrane.

High Zeta values shows stability of the cell membrane. As the value reduces with the increasing concentration of nisin, it shows that nisin interferes with membrane deformation which will lead to reduction of membrane stability.

Zeta potential value is also dependant on pH values. Higher Zeta value shows acidic environment of the membrane. As *Bacillus subtili*s is a Gram positive bacterial presence of teichoic acid in the cell membrane which serves as a chelating agent, makes the environment acidic. When cells are treated with nisin, the cell membrane gets ruptured and that leads to disruption of compact teichoic acid layer. That will show a lower Zeta value.

4.6 Scanning Electron Microscopy (SEM) (at 10,000 magnification):

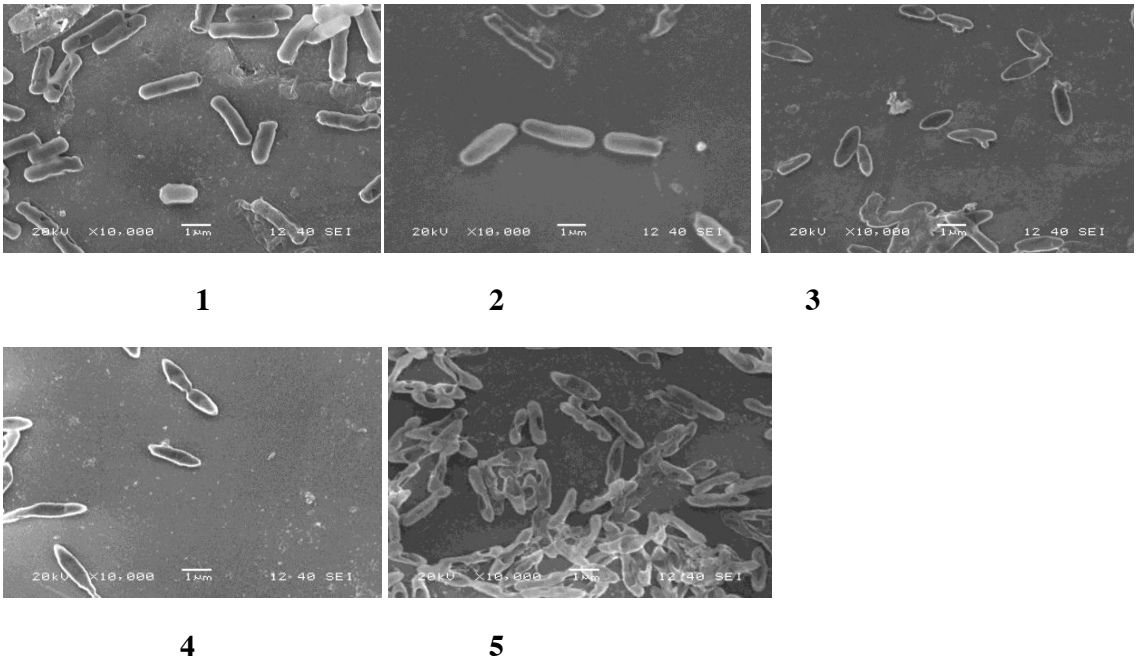
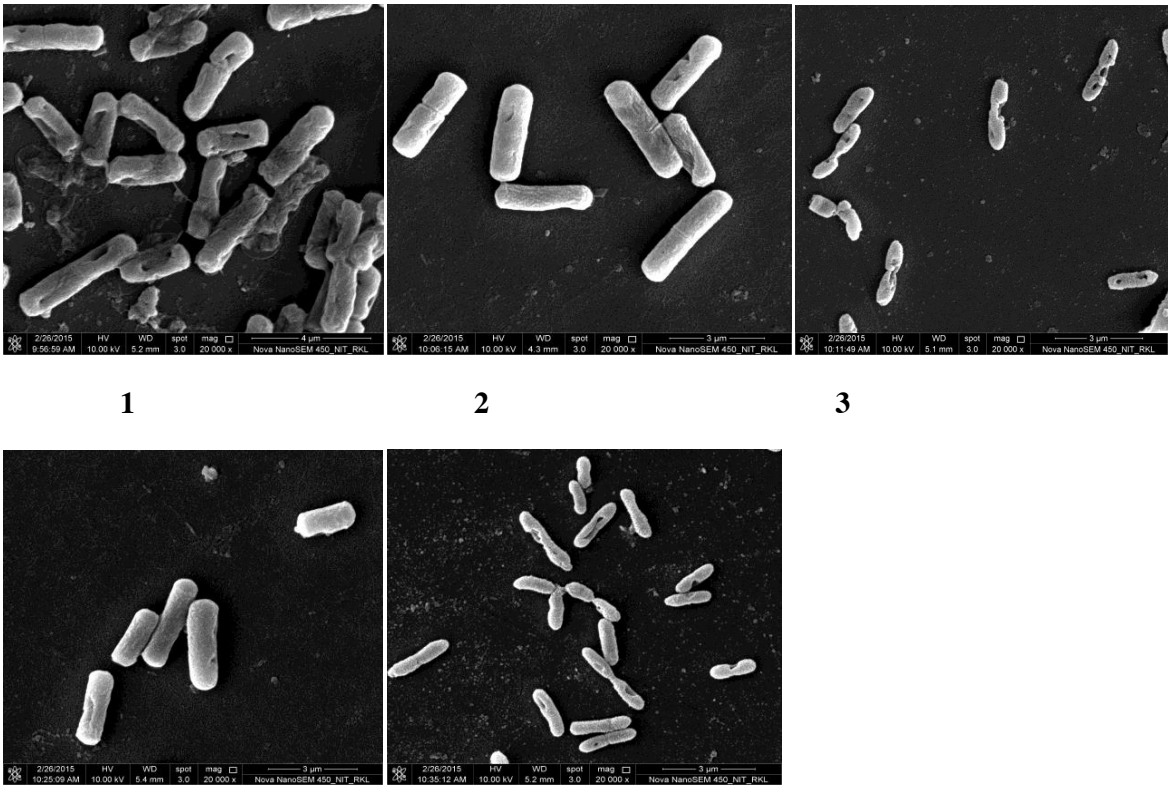


Figure 13: Scanning electron microscopy images 1(control), 2 (Nisin 50U/ml) and 3 (Nisin 1500U/ml) show that with the increasing concentration of nisin, the shape and size of the cells get deformed. In image 4 (Ampicilling Control), the cells are normal but in case of image 5 (Ampicilling +Nisin 1500U/ml), rupture of bacterial cell membrane is observed.

4.7 Field Emission Scanning Electron Microscopy (FESEM) (at 20,000 magnification):



1 **2** **3**

4 **5**

Figure 14: Field Emission Scanning electron microscopy images 1(control), 2 (Nisin 50U/ml) and 3 (Nisin 1500U/ml) show that with the increasing concentration of nisin, the shape and size of the cells get deformed. In image 4 (Ampicilling Control), the cells are normal but in case of image 5 (Ampicilling +Nisin 1500U/ml), rupture of bacterial cell membrane is observed

Scanning Electron Microscopy (At 10,000 magnification):

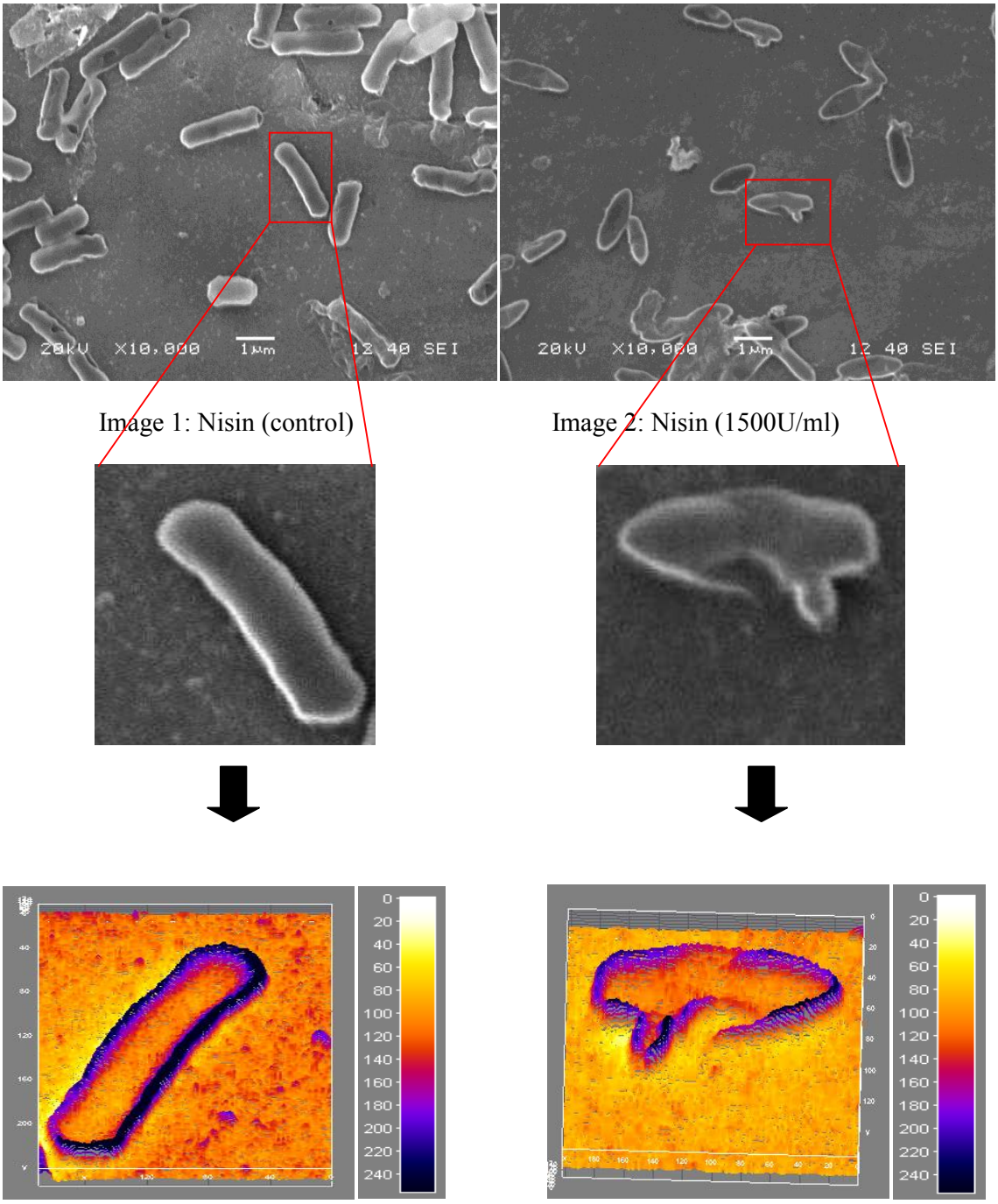


Image 1: Nisin (control) Image 2: Nisin (1500U/ml)

Figure 15: Thermal LUT false color maps of the surface morphology plot of *B subtil*is treated with Nisin (Control and 1500 U/ml)

Scanning Electron Microscopy (At 10,000 magnification):

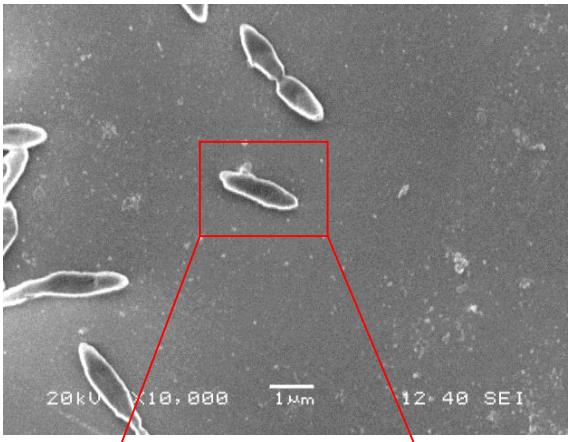


Image 3: Ampicillin + Nisin (Control)

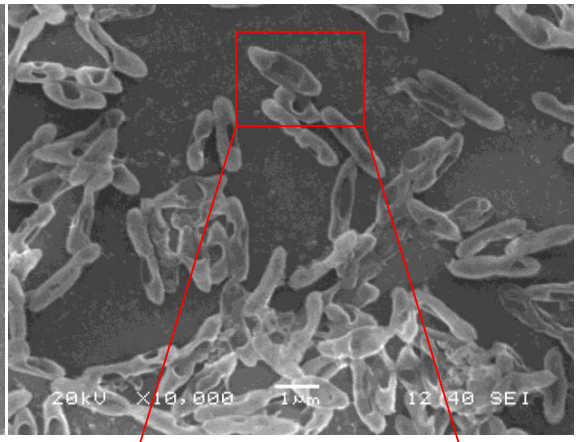
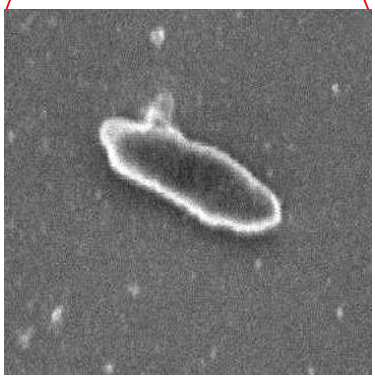
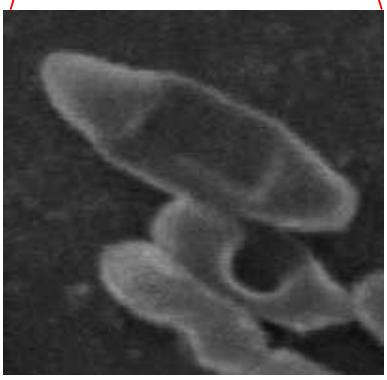
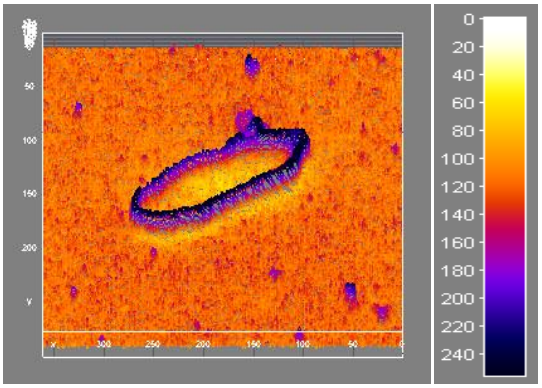


Image 4: Ampicillin + Nisin (1500 U/ml)







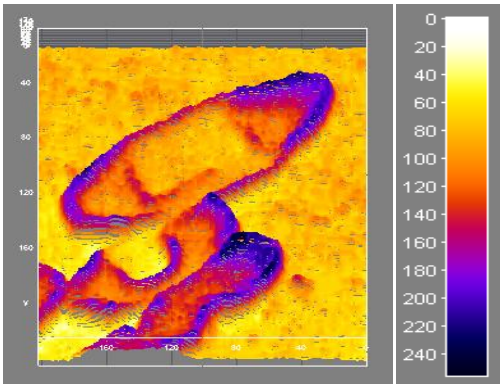


Figure 16: Thermal LUT false color maps of the surface morphology plot of B subtilis treated with Nisin (Control and 1500 U/ml) and Ampicillin.

Field Emission Scanning Electron Microscopy (At 20,000 magnification):

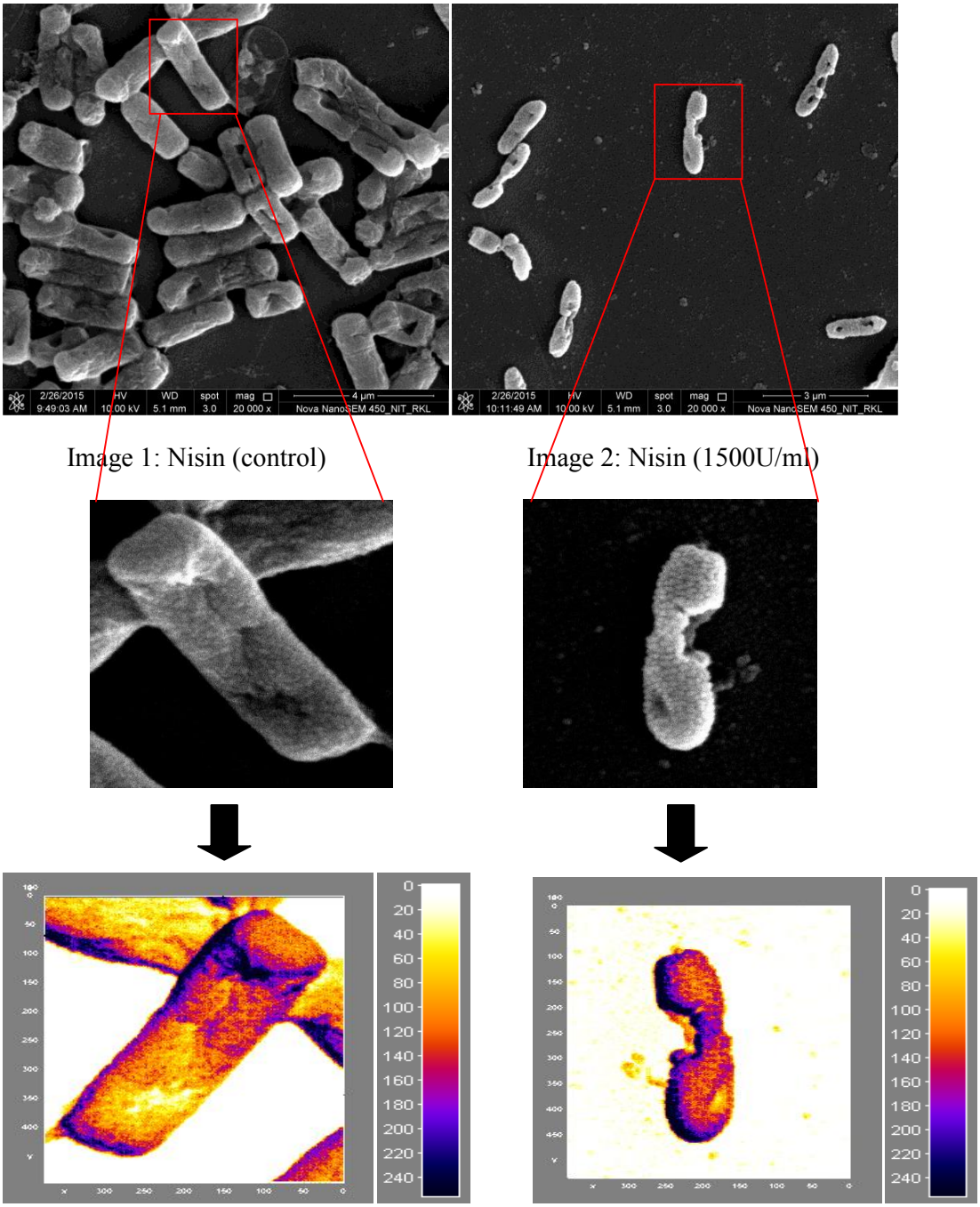


Image 1: Nisin (control)

Image 2: Nisin (1500U/ml)

Figure 17: Thermal LUT false color maps of the surface morphology plot of B subtilis treated with Nisin (Control and 1500 U/ml).

Field Emission Scanning Electron Microscopy (At 20,000 magnification):

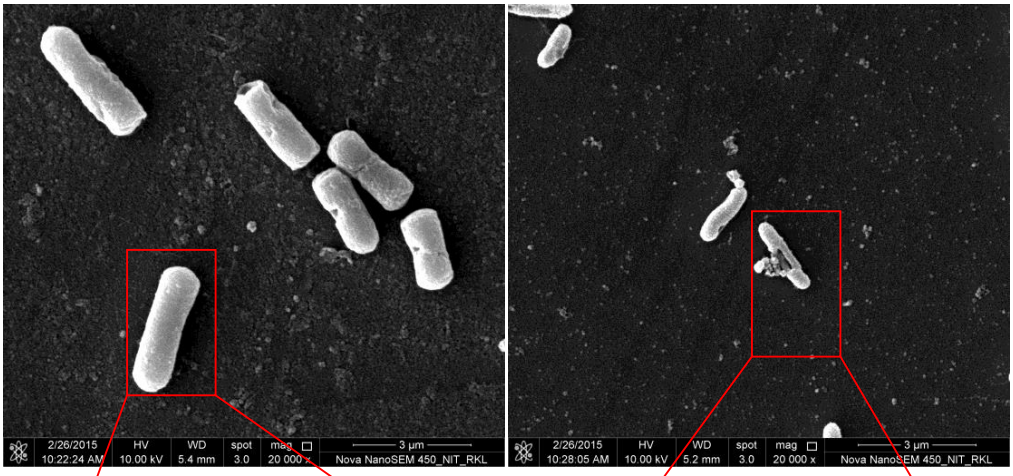


Image 3: Ampicillin + Nisin (control)

Image 4: Ampicillin + Nisin (1500U/ml)

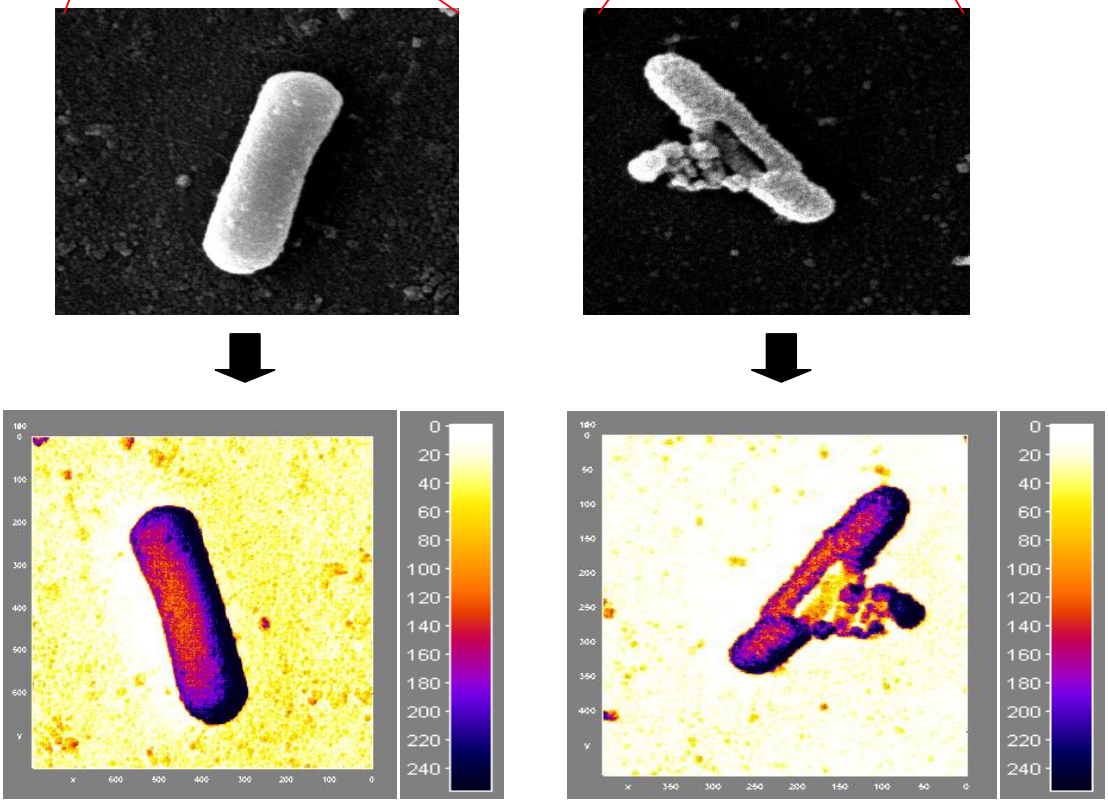


Figure 13: The thermal LUT images above show the deformation of cell when a cell is treated with ampicillin + nisin (1500 U/ml).

4.8 Tabulation (measuring the length, breadth, radius, volume and surface area):

Sl. no	Image	Length (μm)(L)	Breadth (μm)(w)	Radius (μm) (R)	Volume (μm^3) ($V = \frac{\pi}{4}w^2L+\frac{\pi}{3}w^2R$)	Surface area (μm^2) ($A= 2\pi RL+2\pi R^2$)
1	Control	2.82±0.24	0.89±0.13	5.94×10^{-3}	1.74689	0.10616
2	Nisin 50U/ml	2.63±0.23	0.89±0.11	4.72×10^{-3}	1.62871	0.07808
3	Nisin 1500U/ml	1.52±0.14	0.43±0.9	1.36×10^{-3}	0.22036	0.01299
4	Amp Control	3.45±0.39	0.82±0.11	5.70×10^{-3}	1.81340	0.12369
5	Amp+Nisin1500U/ml	1.25±0.17	0.42±0.7	1.31×10^{-3}	0.17223	0.01029

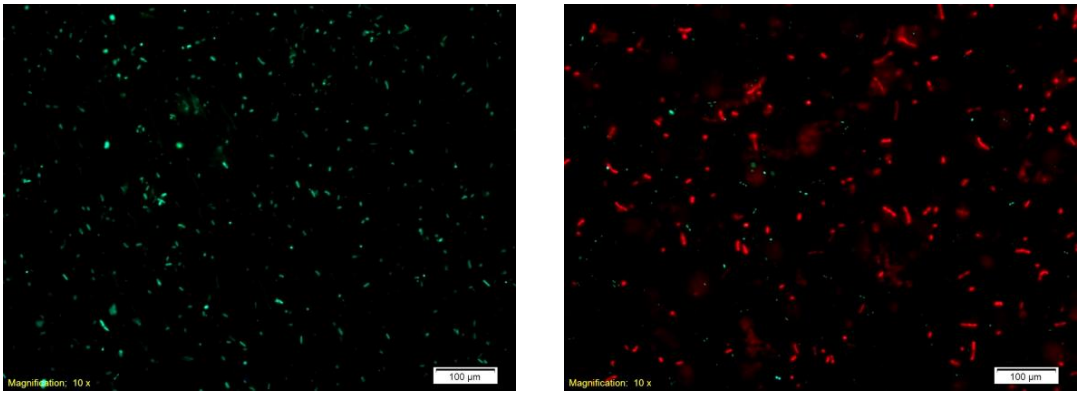
The images on the previous leaves and the tabulation above show that, there is a decrease in length, breadth, radius of the bacterial cell when the nisin concentration is increasing. While the volume and surface area are calculated from the 2D images, they are also decreasing accordingly.

The treatment of nisin leads to tapering of cell in both the sides. That would be the result from damage of cell membrane.

Cell membrane can be deformed by various reasons. Loss of phospholipids from decreased synthesis or increased degradation is an important mechanism to cell damage. As nisin reduces the production of necessary enzymes, DNA, RNA etc., that may lead to loss of phospholipids which may taper the cells.

The cytoskeleton composed of microfilaments, intermediate filaments and microtubules serves as a structural support system and transport system for the cell. Detachment of the cytoskeleton from the plasma membrane is caused by nisin intoxication which results in membrane deformation.

BacLight:



1: Nisin (control) 2: Nisin (1500 U/ml)

Fig 14: BacLight images: Image 1 nisin (control), Image 2 -nisin (1500 U/ml)

The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes.

The green colour in the image 1 shows that at nisin (control), the cells are alive, and in image 2, the red colour indicates most of the cells are dead due to the incorporation of nisin (1500U/ml).

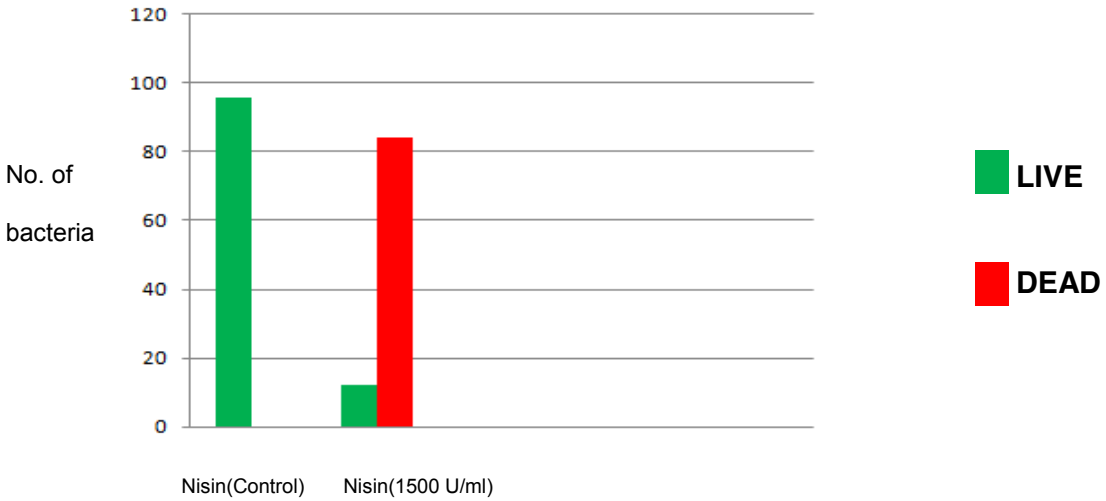


Fig 15: A histogram showing number of live or dead bacteria in nisin control and treated condition

From a count of 96 bacteria in nisin (control) condition, only 12 bacteria live when the concentration of nisin ic increased to 1500 U/ml. Hence, it suggests 83.5 % bacteria die in treated (nisin 1500 U/ml) codition.

Chapter 5
DISCUSSION

In the present study, we investigate the morphological changes in B. subtilis, a Gram-positive rod, after incubation with nisin to analyze the effects of the antibiotic during its bactericidal action in vivo.

In MIC test it is observed that with increasing concentration of nisin, the prolongation of lag phase occurs. This may reflect that nisin reduces the ability of the bacterial cells to stick to the lag phase, where the cell increases its size and makes itself ready to divide by synthesizing necessary enzymes, DNA, RNA etc.

The decrease in the number of bacterial colonies in MBC test suggests, higher concentration of nisin leads to cell death.

The Zeta potential result shows that the deformation of cell membrane causes the reduction of teichoic acid because *B. subtilis* is a Gram positive bacterium. The acidity of the medium is reduced so that the Zeta value increases showing membrane instability after incorporation of nisin.

The images from electron microscopy show reduction in cell length, which indicates that cell-wall inhibition occurs mainly during the active cell elongation of bacteria division cycle. The tapering of cell may be caused due to loss of phospholipids from the cell membrane. Also the cytoskeleton may get detached from the cell membrane, so that the membrane loses its elasticity and gets tapered.

Chapter 6
CONCLUSION

Nisin induced leakage of cytoplasmic contents from treated samples, It seems to hinder growth of *B*. *subtilis*. The cell division of the bacteria is drastically slowed down and the shape and size get significantly affected that changes the cylindrical cells to tapered ones.

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